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(54) Title: **DELTA 6 FATTY ACID DESATURASE**

(57) Abstract

Novel human DNA sequences that encode the gene CYB5RP, a delta 6 fatty acid desaturase, are provided. Provided are genomic CYB5RP DNA as well as cDNA that encodes the CYB5RP protein. Also provided is CYB5RP protein encoded by the novel DNA sequences. Methods of expressing CYB5RP protein in recombinant systems are provided. Also provided are CYB5RP methods that identify activators and inhibitors of CYB5RP protein.

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TITLE OF THE INVENTION
DELTA 6 FATTY ACID DESATURASE

CROSS-REFERENCE TO RELATED APPLICATIONS

5 Not applicable.

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not applicable.

10 REFERENCE TO MICROFICHE APPENDIX

Not applicable.

FIELD OF THE INVENTION

15 The present invention is directed to novel human DNA sequences encoding a delta 6 fatty acid desaturase, an enzyme involved in the synthesis of essential fatty acids.

BACKGROUND OF THE INVENTION

20 Essential fatty acids (EFAs) are polyunsaturated fatty acids that cannot be manufactured by mammals, yet are required for a number of important biochemical processes, and thus must be supplied in the diet. The most important dietary EFAs are linoleic acid and alpha-linolenic acid (ALA). These two EFAs undergo a number of biosynthetic reactions that convert them into various other EFAs. Figure 1 depicts the biosynthetic reactions involving the two groups of EFAs, the n-6 EFAs (linoleic acid derivatives) and the n-3 EFAs (ALA derivatives). EFAs are formed from linoleic acid and ALA by a series of alternating reactions involving the removal of two hydrogens coupled with the insertion of an additional double bond (desaturation) and the lengthening of the fatty acid chain by the addition of two carbons (chain elongation). The enzymes catalyzing the desaturations and elongations are thought to 25 be the same for both groups of EFAs.

30 Among the more important unsaturated fatty acids are the delta 6 unsaturated fatty acids, which are involved in the maintenance of membrane structure and function, the regulation of cholesterol synthesis and transport, and the prevention

of water loss from the skin. Delta 6 unsaturated fatty acids also serve as precursors of the eicosanoids, including the prostaglandins and leukotrienes (Horrobin, 1992, *Prog. Lipid Res.* 31:163-194). The double bond at the 6 position of delta 6 unsaturated fatty acids is introduced by a class of enzymes known as delta 6 desaturases.

5 Deficiencies in linoleic acid and ALA derivatives have been associated with skin diseases, diabetic complications, inflammatory and autoimmune disorders, cardiovascular disorders, complications of viral infection, and retinal dysfunction. For example, a deficiency in gamma-linolenic acid (GLA), which is produced from linoleic acid by the action of the enzyme delta 6 desaturase, can arise from the 10 decreased activity of this enzyme that occurs in aging, stress, diabetes, eczema, and some infections, or from increased catabolism of GLA due to oxidation or rapid cell division, as occurs in inflammation or cancer. Clinical trials have demonstrated that dietary GLA supplementation can be effective in treating a number of conditions that are associated with GLA deficiency, *e.g.*, atopic eczema, mastalgia, diabetic 15 neuropathy, viral infections, and some forms of cancer (Horrobin, 1990, *Rev. Contemp. Pharmacother.* 1:1-45).

Delta 6 desaturase is an example of a fatty acid desaturase. Fatty acid desaturases are enzymes that introduce a double bond into the carbon chain of fatty acids. They play vital roles in the biosynthesis of polyunsaturated fatty acids, 20 including the essential fatty acids. Fatty acid desaturases are present in soluble and membrane-associated forms and require electron donors (for example, cytochrome b5) for their functioning.

Delta 6 desaturases catalyze the rate-limiting steps in the biosyntheses of the linoleic and ALA group EFAs shown in Figure 1. End products of the linoleic acid pathway include the eicosanoids (prostaglandins and leukotrienes). The end 25 product of the ALA pathway is docosahexaenoic acid (DHA), an important component of membranes in the vertebrate retina. DHA is highly specific for retina and represents more than 50% of the fatty acids in the rod outer segment (ROS). It appears that DHA is important in maintaining the normal structure and function of the 30 retina (Anderson et al., 1992, *Neurobiology of Essential Fatty Acids*, Bazan et al., eds., Plenum Press, New York, pages 285-294). Increased dietary consumption of DHA and its precursor, eicosapentaenoic acid, from seal meat and fish has been

linked to an increased incidence of macular degeneration in Greenland Eskimos (Rosenberg, 1987, *Arct. Med. Res.* 46:64-70).

Certain delta 6 desaturases have been cloned from plants. For example, a delta 6 desaturase has been cloned from borage (Sayanova et al., 1997, 5 *Proc. Natl. Acad. Sci. USA* 94:4211-4216). This delta 6 desaturase is unusual in that its cytochrome b5 electron donor is present as an N-terminal extension of the enzyme rather than being synthesized as a separate protein. The borage delta 6 desaturase has been shown to be functional, in that transfer of the cloned gene encoding it to tobacco results in the synthesis of high levels of GLA and octadecatetraenoic acid (OTA) in 10 the transgenic tobacco leaves. GLA and OTA are the products of delta 6 desaturase activity on linoleic acid and ALA, respectively.

Based on its hydrophy profile, the borage delta 6 desaturase appears to be a membrane-bound protein. Examination of the amino acid sequence of the borage enzyme, as well as the amino acid sequences of membrane-bound desaturases 15 from a wide variety of organisms, has revealed three regions of conserved short motifs containing histidine residues (HX(3 or 4)H, HX(2 or 3)HH, and HX(2 or 3)HH) having a conserved spacing from each other (Shanklin et al., *Biochemistry*, 1994, 33:12787-12794).

A DNA sequence has been isolated from sunflower embryos that, 20 judging from its sequence, appears to encode a delta 6 desaturase having a cytochrome b5-like moiety fused to its N-terminus (Sperling et al., 1995, *Eur. J. Biochem.* 232:798-805).

SUMMARY OF THE INVENTION

25 The present invention is directed to novel human DNA sequences that encode a delta 6 fatty acid desaturase, cytochrome b5-related protein (CYB5RP). The present invention includes genomic CYB5RP DNA as well as cDNA that encodes the CYB5RP protein. The genomic CYB5RP DNA is substantially free from other nucleic acids and has the nucleotide sequence shown in SEQ.ID.NO.:1. The cDNA 30 encoding CYB5RP protein is substantially free from other nucleic acids and has the nucleotide sequence shown in SEQ.ID.NO.:2. Also provided is CYB5RP protein encoded by the novel DNA sequences. The CYB5RP protein is substantially free from other proteins and has the amino acid sequence shown in SEQ.ID.NO.:3.

Methods of expressing CYB5RP protein in recombinant systems are provided. Also provided are methods of producing delta 6 unsaturated fatty acids using DNA encoding CYB5RP or using CYB5RP protein.

5 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the enzymatic conversions involved in the linoleic acid (n-3) and alpha-linolenic acid (n-6) pathways of essential fatty acid synthesis.

Figure 2A-G shows the genomic DNA sequence of the CYB5RP gene (SEQ.ID.NO.:1). Underlined nucleotides in capitals represent exons. The start ATG codon at position 544 in exon 1 and the stop TGA codon at position 18,103 in exon 12 are shown in bold. The putative polyadenylation signal ATTAAA located approximately 20 base pairs upstream of the polyA tail is shown in bold italics (position 18,373 in exon 12). DNA sequence upstream of exon 1 represents a putative promoter region of the CYB5RP gene., as indicated by the presence of the 10 TATA box at position 353 (underlined bold)..

Figure 3A-C shows the cDNA sequence (SEQ.ID.NO.:2) and the amino acid sequence (SEQ.ID.NO.:3) of CYB5RP. The region encompassing amino acids 1-102 represents the cytochrome b5 domain. The region encompassing amino acids 182-186 represents HIS BOX 1. The region encompassing amino acids 219-223 represents HIS BOX 2. The region encompassing amino acids 383-387 represents HIS BOX 3.

Figure 4 shows a portion of the cDNA sequence (SEQ.ID.NO.:4) and a portion of the amino acid sequence (SEQ.ID.NO.:5) of mouse CYB5RP.

Figure 5A shows a Kyte-Doolittle hydropathy plot of CYB5RP. Figure 5B shows the proposed membrane topology of CYB5RP based on its hydropathy plot. This membrane topology is similar to that proposed for other membrane-bound fatty acid desaturases (Shanklin et al., Biochemistry, 1994, 33:12787-12794). The amino acids shown in Figure 5B are portions of (SEQ.ID.NO.:3).

Figure 6 shows the output of the Profilescan program from the Wisconsin GCG package. The upper amino acid sequence is from CYB5RP (positions 31-78 of SEQ. ID. NO.3). The lower amino acid sequence is positions 1-48 of the cytochrome b5 profile (SEQ. ID. NO.:6.). The output shows that CYB5RP

contains a profile typical for the heme-binding domain of the cytochrome b5 protein family. Importantly, the region of identity includes the invariant HPGG motif, where histidine represents a heme axial ligand for iron.

Figure 7A and B show the results of BlastP searches of the GenBank database using the full-length CYB5RP amino acid sequence as the query. Figure 7A shows the hit with highest homology, a hypothetical protein from sunflower. The sunflower protein and CYB5RP share three His boxes (boxed) in which the spacing between the His boxes is conserved. Also boxed is the HPGG motif typical for the heme-binding domain of the cytochrome b5 protein family. In both proteins the first histidine of the third His box is replaced by glutamine (a typical feature of desaturases with delta 6 specificity). The upper amino acid sequences shown are from CYB5RP and are portions of SEQ. ID. NO.3. The lower amino acid sequences shown are portions of the amino acid sequence of the hypothetical protein from sunflower (Sperling et al., 1995, Eur. J. Biochem. 232:798-805). The sequence shown as positions 348-432 is SEQ. ID. NO.:7. The sequence shown as positions 22-74 is SEQ. ID. NO.:8. The sequence shown as positions 152-227 is SEQ. ID. NO.:9. Figure 7B shows the hit with the second highest homology, a delta 6 desaturase from *Borago officinalis* (Sayanova et al., 1997, Proc. Natl. Acad. Sci. USA 94:4211-4216). The *Borago* protein and CYB5RP also share three His boxes with conserved spacing, as well as the HPGG motif. In both proteins the first histidine of the third His box is replaced by glutamine (a typical feature of desaturases with delta 6 specificity). The upper amino acid sequences shown are from CYB5RP and are portions of SEQ. ID. NO.3. The lower amino acid sequences shown are portions of the amino acid sequence of the *Borago* delta 6 desaturase. The sequence shown as positions 338-424 is SEQ. ID. NO.:10. The sequence shown as positions 12-64 is SEQ. ID. NO.:11. The sequence shown as positions 153-220 is SEQ. ID. NO.:12.

Figure 8 shows additional results of BlastP searches of the GenBank database using the CYB5RP protein as the query. Figure 8 shows the amino acid alignment between the CYB5RP protein and a delta 6 desaturase from *Synechocystis* sp. (strain pcc 6803) performed by the BlastP program. The *Synechocystis* delta 6 desaturase and CYB5RP share three His boxes, two of which are shown in Figure 8 (boxed). In both proteins the first histidine of the third His box is replaced by glutamine (a typical feature of desaturases with delta 6 specificity). The CYB5RP

sequence shown is a portion of SEQ. ID. NO.3. The *Synechocystis* sequence shown is SEQ. ID. NO:13.

Figure 9A shows the expression pattern of the CYB5RP gene in 9 human tissues, as determined by RT-PCR amplification with 21 cycles. Expression is 5 detected in human retina, kidney, pancreas, placenta, and brain. Figure 9B shows the results of the analogous experiments performed with 25 cycles of amplification. Expression of the CYB5RP gene is seen in all the human tissues studied.

DETAILED DESCRIPTION OF THE INVENTION

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For the purposes of this invention:

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“Substantially free from other proteins” means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other proteins. Thus, a CYB5RP protein preparation that is substantially free from other proteins will contain, as a percent of its total protein, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of non-CYB5RP proteins. Whether a given CYB5RP protein preparation is substantially free from other proteins can be determined by such conventional techniques of assessing protein purity as, *e.g.*, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) combined with appropriate detection methods, *e.g.*, 20 silver staining or immunoblotting.

25

“Substantially free from other nucleic acids” means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other nucleic acids. Thus, a CYB5RP DNA preparation that is substantially free from other nucleic acids will contain, as a percent of its total nucleic acid, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of non-CYB5RP nucleic acids. Whether a given CYB5RP DNA preparation is substantially free from other nucleic acids can be determined by such conventional techniques of assessing nucleic acid purity as, *e.g.*, agarose gel electrophoresis combined with appropriate staining methods, *e.g.*, 30 ethidium bromide staining, or by sequencing.

35

“Substantially the same biological activity as CYB5RP” means being able to introduce a double bond into the 6 position of linoleic acid under conditions in which CYB5RP is able to introduce a double bond into the 6 position of linoleic acid.

A "conservative amino acid substitution" refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples of such conservative substitutions are: substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (e.g., arginine for lysine; glutamic acid for aspartic acid); substitution of one aromatic amino acid (tryptophan, tyrosine, or phenylalanine) for another.

The present invention relates to the identification and cloning of cytochrome b5-related protein (CYB5RP), a gene which encodes a human delta 6 fatty acid desaturase. The gene is present on PAC clones 759J12, 756B3, 519O13, and 466A11 from an area of human chromosome 11q12 that has been shown to contain a gene related to Best's macular dystrophy (Cooper *et al.*, 1997, *Genomics* 41:185-192; Stöhr *et al.*, 1997, *Genome Res.* 8:48-56; Graff *et al.*, 1997, *Hum. Genet.* 101: 263-279). This linkage between the chromosomal location of the CYB5RP gene and the location of the gene related to Best's macular dystrophy can be used diagnostically by identifying restriction fragment length polymorphisms (RFLPs) in the vicinity of the CYB5RP gene, e.g., in SEQ.ID.NO.:1. Such RFLPs will be associated with the Best's macular dystrophy gene and thus can be used to identify individuals carrying disease-causing forms of the Best's macular dystrophy gene.

CYB5RP was identified as an EST hit in sequence scanning data from PAC clones from human chromosome 11q12. In addition, a full length cDNA of CYB5RP was recovered from a human retina cDNA library. The genomic region of CYB5RP has been sequenced and the exon/intron organization of CYB5RP has been determined. The CYB5RP gene has 12 exons. The promoter region of CYB5RP was identified upstream of the 5' UTR by detecting consensus elements required for eukaryotic transcription. The expression pattern of CYB5RP was determined by RT-PCR analysis in 9 human tissues. The CYB5RP gene is expressed predominantly in human retina, kidney, pancreas, and placenta; lower levels of expression are also detected in brain, heart, lung, liver, and skeletal muscle. Bioinformatic analysis revealed significant homology to a group of plant and bacterial fatty acid desaturases. All of the typical amino acid motifs present in these fatty acid desaturases are also present in CYB5RP. Kyte-Doolittle algorithm analysis predicts a transmembrane organization typical of fatty acid desaturases for CYB5RP (see Figure 5). CYB5RP is

unusual in that it contains a cytochrome b5 region in its N terminus. While many fatty acid desaturases utilize cytochrome b5 as an electron donor, most have not incorporated this cytochrome as part of their polypeptide chain.

That CYB5RP is a fatty acid desaturase is shown by the following 5 evidence:

- (1) CYB5RP possesses significant homology to a group of plant and microbial fatty acid desaturases;
- (2) Like other fatty acid desaturases, CYB5RP has three conserved histidine boxes, with correct spacing between the boxes; and
- 10 (3) The predicted membrane topology of CYB5RP is similar to that of known fatty acid desaturases.

That CYB5RP is a delta 6 fatty acid desaturase is shown by the following evidence:

- 15 (1) CYB5RP contains a cytochrome b5-like moiety fused to its N-terminus. The only two fatty acid desaturases that contain cytochrome b5-like moiety fused to their N-termini are known or suspected to be delta 6 desaturases.
- (2) The only two plant desaturases that are known or suspected to introduce a double bond in the 6 position have an atypical His box 3 (QI/LEHH), with a Q in the first position rather than an H. CYB5RP has the same atypical His Box 3.
- 20 (3) The only bacterial desaturase that is known to introduce a double bond in the 6 position has an atypical His box 3 (QVTHH), with a Q in the first position rather than an H. CYB5RP has the same atypical His Box 3.

CYB5RP is a target for the development of drugs for the treatment of disorders of lipid metabolism and for the treatment of conditions that require the 25 modulation of the biosynthesis of prostaglandins and leukotrienes (asthma, pain, *etc.*). CYB5RP is also a target for the development of drugs for use in treating skin diseases, diabetic complications, reproductive disorders, including breast pain and premenstrual syndrome, inflammatory and autoimmune disorders, cardiovascular disorders, complications of viral infections, and various forms of retinal degeneration, 30 including age-related macular degeneration.

CYB5RP is homologous to a delta 6 desaturase from *Borago officinalis* (see Figure 7B). Both CYB5RP and this *Borago* delta 6 desaturase, unlike desaturases from higher plants, are unusual in containing a cytochrome b5-like

domain fused to their N-termini (Sayanova et al., 1997, Proc. Natl. Acad. Sci. USA 94:4211-4216; hereinafter "Sayanova"). The *Borago* desaturase has been expressed in transgenic tobacco, resulting in high levels of delta 6 desaturated fatty acids in the transgenic tobacco leaves, including high levels of γ -linolenic acid (GLA) (Sayanova).

5 Given the medical importance of GLA, Sayanova proposed that transgenic plants, expressing the *Borago* delta 6 desaturase, would be valuable as sources of GLA. Similarly, CYB5RP, expressed in transgenic plants, is expected to provide a valuable source of GLA.

The present invention provides DNA encoding CYB5RP that is substantially free from other nucleic acids. The present invention also provides recombinant DNA molecules encoding CYB5RP. The present invention provides DNA molecules substantially free from other nucleic acids comprising the nucleotide sequence shown in Figure 2 as SEQ.ID.NO.:1. Analysis of SEQ.ID.NO.:1 revealed that this genomic sequence defines a gene having 12 exons. These exons collectively have an open reading frame that encodes a protein of 445 amino acids. When an alternatively spliced exon 8 is used, a CYB5RP protein of 433 amino acids, lacking amino acids 317-328, is produced. Thus, the present invention includes two cDNA molecules, encoding two forms of CYB5RP protein, that are substantially free from other nucleic acids. The first cDNA is shown in Figure 3 and has the nucleotide sequence SEQ.ID.NO.:2. The second cDNA is identical to the first, except that it does not contain the nucleotides at positions 1,019-1,054.

The present invention includes DNA molecules substantially free from other nucleic acids comprising the coding region of SEQ.ID.NO.:2. Accordingly, the present invention includes DNA molecules substantially free from other nucleic acids having a sequence comprising positions 71-1,405 of SEQ.ID.NO.:2. The present invention also includes DNA molecules substantially free from other nucleic acids having a sequence comprising positions 71-1,405 of SEQ.ID.NO.:2, except that the nucleotides at positions 1,019-1,054 are missing. Also included in the present invention are recombinant DNA molecules having a nucleotide sequence comprising positions 71-1,405 of SEQ.ID.NO.:2 and recombinant DNA molecules having a nucleotide sequence comprising positions 71-1,405 of SEQ.ID.NO.:2 with the exception that positions 1,019-1,054 are missing.

The novel DNA sequences of the present invention encoding CYB5RP, in whole or in part, can be linked with other DNA sequences, *i.e.*, DNA sequences to which CYB5RP is not naturally linked, to form "recombinant DNA molecules" encoding CYB5RP. Such other sequences can include DNA sequences that control transcription or translation such as, *e.g.*, translation initiation sequences, promoters for RNA polymerase II, transcription or translation termination sequences, enhancer sequences, sequences that control replication in microorganisms, sequences that confer antibiotic resistance, or sequences that encode a polypeptide "tag" such as, *e.g.*, a polyhistidine tract or the myc epitope. The novel DNA sequences of the present invention can be inserted into vectors such as plasmids, cosmids, viral vectors, P1 artificial chromosomes, or yeast artificial chromosomes.

Included in the present invention are DNA sequences that hybridize to at least one of SEQ.ID.NOs.:1 or 2 under stringent conditions. By way of example, and not limitation, a procedure using conditions of high stringency is as follows:

15 Prehybridization of filters containing DNA is carried out for 2 hr. to overnight at 65°C in buffer composed of 6X SSC, 5X Denhardt's solution, and 100 µg/ml denatured salmon sperm DNA. Filters are hybridized for 12 to 48 hrs at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 X 10⁶ cpm of ³²P-labeled probe. Washing of filters is done at 37°C for 1 hr in a 20 solution containing 2X SSC, 0.1% SDS. This is followed by a wash in 0.1X SSC, 0.1% SDS at 50°C for 45 min. before autoradiography.

Other procedures using conditions of high stringency would include either a hybridization carried out in 5XSSC, 5X Denhardt's solution, 50% formamide at 42°C for 12 to 48 hours or a washing step carried out in 0.2X SSPE, 0.2% SDS at 25 65°C for 30 to 60 minutes.

Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of these reagents can be found in, *e.g.*, Sambrook, Fritsch, and Maniatis, 1989, Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor 30 Laboratory Press. In addition to the foregoing, other conditions of high stringency which may be used are well known in the art.

The degeneracy of the genetic code is such that, for all but two amino acids, more than a single codon encodes a particular amino acid. This allows for the

construction of synthetic DNA that encodes the CYB5RP protein where the nucleotide sequence of the synthetic DNA differs significantly from the nucleotide sequence of SEQ.ID.NO.:2, but still encodes the same CYB5RP protein shown as SEQ.ID.NO.:3. Such synthetic DNAs are intended to be within the scope of the 5 present invention. Also with the scope of the present invention are synthetic DNAs that encode a CYB5RP protein lacking amino acids 317-328 of SEQ.ID.NO.:3.

Another aspect of the present invention includes host cells that have been engineered to contain and/or express DNA sequences encoding CYB5RP protein. Such recombinant host cells can be cultured under suitable conditions to 10 produce CYB5RP protein. An expression vector containing DNA encoding CYB5RP protein can be used for expression of CYB5RP protein in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of human, bovine, porcine, monkey and rodent origin, plant cells 15 such as tobacco, and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Cell lines derived from mammalian species which are suitable for recombinant expression of CYB5RP protein and which are commercially available, include but are not limited to, L cells L-M(TK⁻) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171).

A variety of mammalian expression vectors can be used to express recombinant CYB5RP in mammalian cells. Commercially available mammalian 25 expression vectors which are suitable include, but are not limited to, pMC1neo (Stratagene), pSG5 (Stratagene), pcDNAI and pcDNAIamp, pcDNA3, pcDNA3.1, pCR3.1 (Invitrogen), EBO-pSV2-neo (ATCC 37593), pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), and pSV2-dhfr (ATCC 37146). Following expression in 30 recombinant cells, CYB5RP can be purified by conventional techniques to a level that is substantially free from other proteins. A description of vectors that can be used to express CYB5RP can be found in, e.g., Goeddel, ed., 1990, Meth. Enzymol. vol. 185 or Perbal, 1988, A Practical Guide to Molecular Cloning, John Wiley and Sons, Inc.

The present invention includes CYB5RP protein substantially free from other proteins. The amino acid sequence of the full-length CYB5RP protein is shown in Figure 3 as SEQ.ID.NO.:3. Thus, the present invention includes CYB5RP protein substantially free from other proteins having the amino acid sequence SEQ.ID.NO.:3. Also included in the present invention is a CYB5RP protein that is produced from an alternatively spliced CYB5RP mRNA where the protein has the amino acid sequence of SEQ.ID.NO.:3 with the exception that amino acids 317-328 are missing.

As with many proteins, it is possible to modify many of the amino acids of CYB5RP and still retain substantially the same biological activity as the original protein. Thus, the present invention includes modified CYB5RP proteins which have amino acid deletions, additions, or substitutions but that still retain substantially the same biological activity as CYB5RP. It is generally accepted that single amino acid substitutions do not usually alter the biological activity of a protein (see, e.g., Molecular Biology of the Gene, Watson *et al.*, 1987, Fourth Ed., The Benjamin/Cummings Publishing Co., Inc., page 226; and Cunningham & Wells, 1989, *Science* 244:1081-1085). Accordingly, the present invention includes polypeptides where one amino acid substitution has been made in SEQ.ID.NO.:3 wherein the polypeptides still retain substantially the same biological activity as CYB5RP. The present invention also includes polypeptides where two or more amino acid substitutions have been made in SEQ.ID.NO.:3 wherein the polypeptides still retain substantially the same biological activity as CYB5RP. In particular, the present invention includes embodiments where the above-described substitutions are conservative substitutions. In particular, the present invention includes embodiments where the above-described substitutions do not occur in the His boxes of CYB5RP. In particular, the present invention includes embodiments where the above-described substitutions do not occur in positions where the amino acid present in those positions in CYB5RP is the same as the amino acid present in the corresponding position of the sunflower protein depicted in Figure 1 of Sperling *et al.*, 1995, *Eur. J. Biochem.* 232:798-805 when these two proteins are aligned by BLASTP analysis. In particular, the present invention includes embodiments where the above-described substitutions do not occur in positions where the amino acid present in those positions in CYB5RP is the same as the amino acid present in the corresponding position of the

CCCTCTACCCCTGTCCCATCAGGC (SEQ.ID.NO.:15)

One of skill in the art would recognize that many other primer pairs based upon SEQ.ID.NO.:2 would also be suitable.

PCR reactions can be carried out with a variety of thermostable enzymes including but not limited to AmpliTaq, AmpliTaq Gold, or Vent polymerase. For AmpliTaq, reactions can be carried out in 10 mM Tris-Cl, pH 8.3, 2.0 mM MgCl₂, 200 μM for each dNTP, 50 mM KCl, 0.2 μM for each primer, 10 ng of DNA template, 0.05 units/μl of AmpliTaq. The reactions are heated at 95°C for 3 minutes and then cycled 35 times using the cycling parameters of 95°C, 20 seconds, 62°C, 20 seconds, 72°C, 3 minutes. In addition to these conditions, a variety of suitable PCR protocols can be found in PCR Primer, A Laboratory Manual, edited by C.W. Dieffenbach and G.S. Dveksler, 1995, Cold Spring Harbor Laboratory Press; or PCR Protocols: A Guide to Methods and Applications, Michael *et al.*, eds., 1990, Academic Press.

A suitable cDNA library from which a clone encoding CYB5RP can be isolated would be Human Retina 5'-stretch cDNA library in lambda gt10 or lambda gt11 vectors (catalog numbers HL1143a and HL1132b, Clontech, Palo Alto, CA). The primary clones of such a library can be subdivided into pools with each pool containing approximately 20,000 clones and each pool can be amplified separately.

By this method, a cDNA fragment encoding an open reading frame of either 445 amino acids (SEQ.ID.NO.:3) or an open reading frame of 433 amino acids (SEQ.ID.NO.:3 lacking the amino acids at positions 317-328) can be obtained. This cDNA fragment can be cloned into a suitable cloning vector or expression vector. For example, the fragment can be cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, San Diego, CA). CYB5RP protein can then be produced by transferring an expression vector encoding CYB5RP or portions thereof into a suitable host cell and growing the host cell under appropriate conditions. CYB5RP protein can then be isolated by methods well known in the art.

As an alternative to the above-described PCR method, a cDNA clone encoding CYB5RP can be isolated from a cDNA library using as a probe oligonucleotides specific for CYB5RP and methods well known in the art for screening cDNA libraries with oligonucleotide probes. Such methods are described

in, e.g., Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K., Vol. I, II. Oligonucleotides that are specific for CYB5RP and that can be used to screen cDNA 5 libraries can be readily designed based upon the cDNA sequence of CYB5RP shown in SEQ.ID.NO.:2 and can be synthesized by methods well-known in the art.

Genomic clones containing the CYB5RP gene can be obtained from commercially available human PAC or BAC libraries available from Research Genetics, Huntsville, AL. PAC clones containing the CYB5RP gene (e.g., PAC 10 clones 759J12, 756B3, 519O13, and 466A11) are commercially available from Research Genetics, Huntsville, AL (Catalog number for individual PAC clones is RPCI.C). Alternatively, one may prepare genomic libraries, especially in P1 artificial 15 chromosome vectors, from which genomic clones containing the CYB5RP can be isolated, using probes based upon the CYB5RP sequences disclosed herein. Methods of preparing such libraries are known in the art (Ioannou *et al.*, 1994, *Nature Genet.* 6:84-89).

The present invention also provides oligonucleotide probes, based upon SEQ.ID.NO.:2 that can be used to determine the level of CYB5RP RNA in a sample. In particular, the present invention includes DNA oligonucleotides 20 comprising at least 18 contiguous nucleotides of SEQ.ID.NO.:2. Also provided by the present invention are corresponding RNA oligonucleotides. The DNA or RNA oligonucleotide probes can be packaged in kits.

In addition to the utilities described above, the present invention makes possible the recombinant expression of the CYB5RP protein in various cell types. In 25 particular, it is advantageous to recombinantly express CYB5RP in plant cells. Such expression in plant cells provides a method for the production of high levels of valuable EFAs such as GLA and OTA in the recombinant plant cells. An example of such recombinant expression of a delta 6 fatty acid desaturase, in that case from borage, is described in Sayanova *et al.*, 1997, *Proc. Natl. Acad. Sci. USA* 94:4211-4216 (Sayanova). The recombinant expression of the borage delta 6 desaturase led to 30 the production of high levels of GLA and OTA in the leaves of the tobacco plants in which it was expressed. The procedures described in Sayanova can be easily adapted to express CYB5RP in tobacco, thus providing an additional, useful way to produce

large amounts of valuable EFAs. Known methods of recombinantly expressing genes in other plant species beside tobacco can be used to express CYB5RP in those other species.

5 The present invention also makes possible the development of assays which measure the biological activity of the CYB5RP protein. Such assays using recombinantly expressed CYB5RP protein are especially of interest.

Assays for CYB5RP protein activity can be used to screen libraries of compounds or other sources of compounds to identify compounds that are activators or inhibitors of the activity of CYB5RP protein. Such identified compounds can 10 serve as "leads" for the development of pharmaceuticals that can be used to modulate the activity of CYB5RP in patients suffering from conditions where that activity is abnormal, *e.g.*, skin diseases, diabetic complications, inflammatory and autoimmune disorders, cardiovascular disorders, complications of viral infection, and retinal dysfunction such as macular degeneration.

15 Such assays may comprise:

- (a) recombinantly expressing CYB5RP protein in a host cell;
- (b) measuring the biological activity of the recombinantly expressed CYB5RP protein in the presence and in the absence of a substance suspected of being an activator or an inhibitor of CYB5RP protein;

20 where a change in the biological activity of the recombinantly expressed CYB5RP protein in the presence as compared to the absence of the substance indicates that the substance is an activator or an inhibitor of CYB5RP protein.

In particular embodiments, the biological activity of the recombinantly 25 expressed CYB5RP protein is the ability to introduce a double bond into the 6 position of linoleic acid or alpha-linoleic acid.

In some embodiments, it may be advantageous to insert additional steps between steps (a) and (b). Such additional steps might include lysing the host cell and fractionating its contents in order to partially purify the recombinantly 30 expressed CYB5RP, thus facilitating exposure of the recombinantly expressed CYB5RP to the substance as well as to any substrate used in the assay.

The present invention includes activators and inhibitors identified by the methods described herein as well as pharmaceutical compositions comprising

such activators and inhibitors. The activators and inhibitors are generally combined with pharmaceutically acceptable carriers before use to form pharmaceutical compositions. Examples of such carriers and methods of formulation of pharmaceutical compositions containing activators or inhibitors and carriers can be 5 found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the activator or inhibitor.

Therapeutic or prophylactic compositions are administered to an individual in amounts sufficient to treat or prevent conditions where CYB5RP activity 10 is abnormal. The effective amount can vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration. The appropriate amount can be determined by a skilled physician.

Compositions can be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents can be desirable.

15 The compositions can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compositions can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection.

20 Likewise, they can also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

25 Advantageously, compositions can be administered in a single daily dose, or the total daily dosage can be administered in divided doses of two, three or four times daily. Furthermore, compositions can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the 30 dosage regimen.

The dosage regimen utilizing the compositions is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route

of administration; the renal, hepatic and cardiovascular function of the patient; and the particular composition thereof employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the composition required to prevent, counter or arrest the progress of the condition. Optimal precision 5 in achieving concentrations of composition within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the composition's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a composition.

The present invention also includes antibodies to the CYB5RP protein. 10 Such antibodies may be polyclonal antibodies or monoclonal antibodies. The antibodies of the present invention are raised against the entire CYB5RP protein or against suitable antigenic fragments of the protein that are coupled to suitable carriers, *e.g.*, serum albumin or keyhole limpet hemocyanin, by methods well known in the art. Methods of identifying suitable antigenic fragments of a protein are known in the art. 15 See, *e.g.*, Hopp & Woods, 1981, Proc. Natl. Acad. Sci. USA 78:3824-3828; and Jameson & Wolf, 1988, CABIOS (Computer Applications in the Biosciences) 4:181-186.

For the production of polyclonal antibodies, CYB5RP protein or an 20 antigenic fragment, coupled to a suitable carrier, is injected on a periodic basis into an appropriate non-human host animal such as, *e.g.*, rabbits, sheep, goats, rats, mice. The animals are bled periodically and sera obtained are tested for the presence of antibodies to the injected antigen. The injections can be intramuscular, intraperitoneal, subcutaneous, and the like, and can be accompanied with adjuvant.

For the production of monoclonal antibodies, CYB5RP protein or an 25 antigenic fragment, coupled to a suitable carrier, is injected into an appropriate non-human host animal as above for the production of polyclonal antibodies. In the case of monoclonal antibodies, the animal is generally a mouse. The animal's spleen cells are then immortalized, often by fusion with a myeloma cell, as described in Kohler & Milstein, 1975, Nature 256:495-497. For a fuller description of the production of 30 monoclonal antibodies, see Antibodies: A Laboratory Manual, Harlow & Lane, eds., Cold Spring Harbor Laboratory Press, 1988.

Gene therapy may be used to introduce CYB5RP polypeptides into the cells of target organs, *e.g.*, the pigmented epithelium of the retina or other parts of the

retina. Nucleotides encoding CYB5RP polypeptides can be ligated into viral vectors which mediate transfer of the nucleotides by infection of recipient cells. Suitable viral vectors include retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, and polio virus based vectors. Alternatively, nucleotides encoding CYB5RP polypeptides can be transferred into cells for gene therapy by non-viral techniques including receptor-mediated targeted transfer using ligand-nucleotide conjugates, lipofection, membrane fusion, or direct microinjection. These procedures and variations thereof are suitable for *ex vivo* as well as *in vivo* gene therapy. Gene therapy with CYB5RP polypeptides will be particularly useful for the treatment of diseases where it is beneficial to elevate CYB5RP activity.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED:

1. A recombinant DNA molecule encoding a polypeptide having the amino acid sequence of SEQ.ID.NO.:3.

5

2. A recombinant DNA molecule comprising a nucleotide sequence selected from the group consisting of:

SEQ.ID.NO.:1;

SEQ.ID.NO.:2;

10

SEQ.ID.NO.:2 lacking positions 1,019-1,054;

positions 71-1,405 of SEQ.ID.NO.:2; and

positions 71-1,405 of SEQ.ID.NO.:2 lacking positions 1,019-1,054.

15

3. A DNA molecule that hybridizes under stringent conditions to the DNA molecule of claim 2.

4. An expression vector comprising the DNA of claim 1.

20

5. A recombinant host cell comprising the DNA of claim 1.

6. A CYB5RP protein, substantially free from other proteins, having an amino acid sequence selected from the group consisting of SEQ.ID.NO.:3 and SEQ.ID.NO.:3 lacking positions 317-328.

25

7. The CYB5RP protein of claim 6 containing a single amino acid substitution.

30

8. The CYB5RP protein of claim 7 where the substitution is a conservative substitution.

9. The CYB5RP protein of claim 6 containing amino acid substitutions where the substitutions do not occur in positions where the amino acid

present in CYB5RP at those positions is also present in the corresponding position in the delta 6 desaturase from sunflower when CYB5RP and the delta 6 desaturase from sunflower are aligned by BLASTP analysis or where the substitutions do not occur in positions where the amino acid present in CYB5RP at those positions is also present
5 in the corresponding position in the delta 6 desaturase from *Synechocystis* when CYB5RP and the delta 6 desaturase from *Synechocystis* are aligned by BLASTP analysis or where the substitutions do not occur in positions where the amino acid present in CYB5RP at those positions is also present in the corresponding position in the delta 6 desaturase from borage when CYB5RP and the delta 6 desaturase from
10 borage are aligned by BLASTP analysis.

10. An antibody that binds specifically to the CYB5RP protein of claim 6.

15 11. A DNA or RNA oligonucleotide probe comprising at least 18 contiguous nucleotides of at least one of the sequences of claim 2.

12. A method for determining whether a substance is an activator or an inhibitor of CYB5RP protein comprising:

20 (a) recombinantly expressing the CYB5RP protein of claim 6 in a host cell;

(b) measuring the biological activity of the recombinantly expressed CYB5RP protein in the presence and in the absence of a substance suspected of being an activator or an inhibitor of CYB5RP protein;

25 where a change in the biological activity of the recombinantly expressed CYB5RP protein in the presence as compared to the absence of the substance indicates that the substance is an activator or an inhibitor of CYB5RP protein.

30 13. The method of claim 12 where the biological activity of CYB5RP protein is the ability to introduce a double bond into the 6 position of linoleic acid.

14. A pharmaceutical composition comprising an activator or an inhibitor of CYB5RP.

15. A method of treating macular degeneration comprising
5 administering to a patient an effective amount of the pharmaceutical composition of
claim 14.

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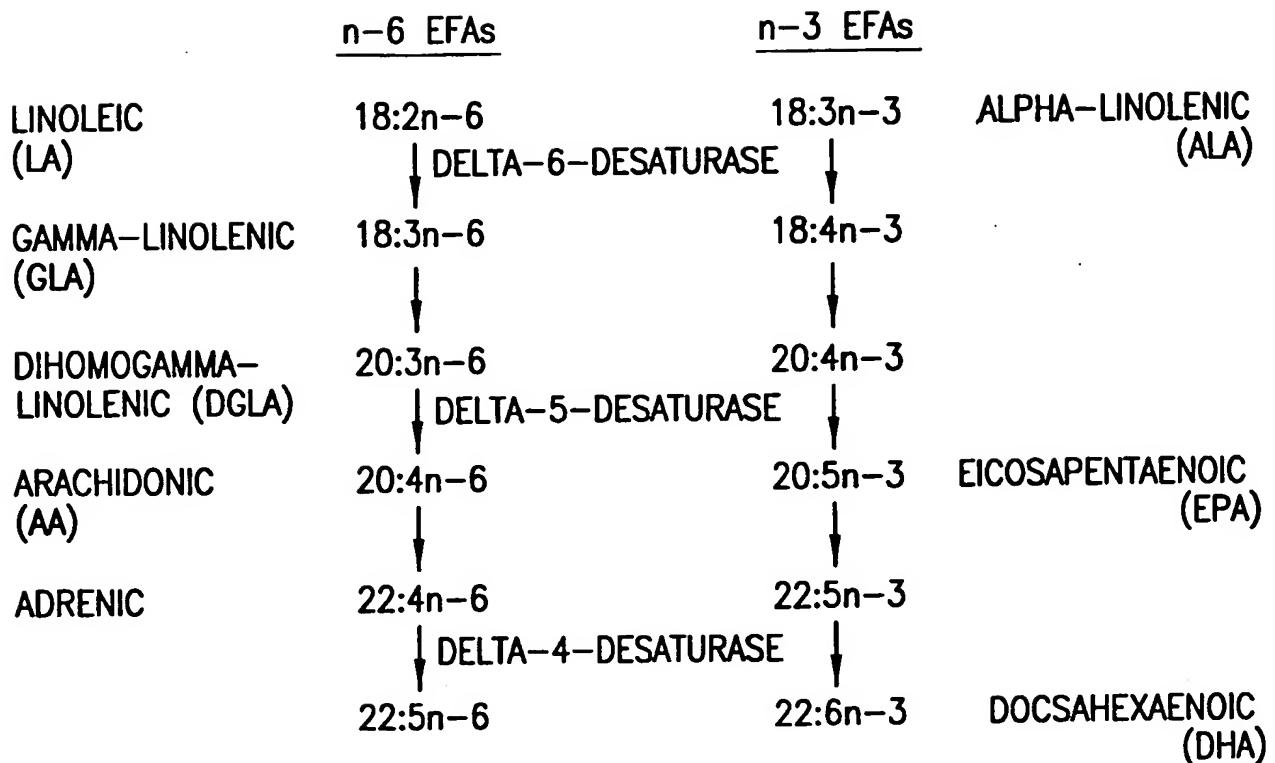


FIG.1

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1 gctcacagac cgggactccg cctccgggttc ccgagggcgt ggcgaggcgc
 51 tgcgggacgc ccaacagggtg cgtgttgtgt ccccaggccc cgcgcctccgg
 101 gtggagtcaa gagcctggaa gccggcagcc cggggaaaagg gggcgggacg
 151 gtgccccggg gcagggctgg gtggcggccg ctgtcctccc gggaggggccc
 201 ggcgcctcg acgcccctt ccctggcggc caatggagac cgaggcccccg
 251 cgcctggatt ggagcggacg cgggggtcaag ccagccttgg gggccggggc
 301 ctggccgggg gcgggggggc aggccggcgc aggccggcgc cgtccgcgc
 351 gttataaaggc ggggagtcc ctgcgcgcgc agccgggagg cgacgcgtcg
 401 ctcgtacggc ggccgcggcg gcagggcggg gccggagcag cggccggcgg
 451 cggaggccgc gcccgggagc gctCTTCGCT TCCCCTGGGG TCTTGCTCGG
 501 ACCTCGGCCA CCGCCTGGGA TCCCCAGGAC TCGTGCCTGC **AGCATGGCG**
 551 GCGTCGGGG GCCGGGACCG CGGGAGGGAC CCGCGCAGCC GGGGGCGCCG
 601 CTGCCCACCT TCTGCTGGGA GCAGATCCGC GCGCACGACC AGCCCAGGCA
 651 CAAGTGGCTG GTCATCGAGC GCCGCGTCTA CGACATCAGC CGCTGGGCAC
 701 AGCGGCACCC AGGGGGCAGC CGCCTCATCG GCCACCACGG CGCTGAGGAC
 751 GCCACGgtaa ggaagccata aggaagccac ccaccggcgg gtggagcctg
 801 gagctcggtc gtgggggtga tggccgcgtc caccgttgg gccttagcat
 851 cctccctccc ctcgctgacc ttgtacctcc acgcccggac ccagagttgg
 901 ggtggactag ccagggccag atgtggggta gggagggcag ttccctgcgt
 951 ggaggacccg cagctgtcca cggagcaggt ctgcggggga ggagggggcc
 1001 tcagagggtgg gtgtgtcatg ctgcagagcc tgccctgggt gaggggctgc
 1051 cctgttgctc ccaggtccct gttcagttc tgggtcccca tgctgggtgc
 1101 ttgctgagtg cttaggggtag ggcagggcag ggtcccccagg ggccggtaag
 1151 gacatgcacat tagaggctgg gggctgggccc ggcctgaggt ctgtggctt
 1201 cccaaagact tctgtaaagg gctcaggggac agtactcac ctctccgggc
 1251 tagcagctgc acgtgggagg gctttgcccag ccaggctggg tgggcctctc
 1301 ctggaagcac agtcacccca ggaacaggct ggcccctggg gaccccaact
 1351 tcccaatccc agccctgtc tagacaggca gggatgttagc ctggccccag
 1401 ggtactgtct ggctggagtc cagtgggtgg gcaagccgcac cagccccctt
 1451 tcccttagtta cccacctgca taataggggt tggggccacg atgcctgtc
 1501 cttgaccctc caaatttcta gttggccac actgggtatc aggaaggtct
 1551 tcaagacccg aggacatgaa tcctgaatgc tggcttttg ggcagcagcg
 1601 gaggttctgt ccagtcccag gactgtcgcc gtcctcttg ccagggccac
 1651 ctgctctctg ccgattgcca tctccagcat gttggacaat cttcaactgg
 1701 ctctttgagg aagaaagccc ctctttccc tttccacccc atgaagctga
 1751 ggagtgagaa taagaatcct cctgaaattc taaaaaaaaa aaaaaaaaaa
 1801 aaagagaacg cttgtccgt ggctgttcag gcccacgc ctggcccgag
 1851 gggacagcac agccgtggaa tgaagcagcc tggggcagt atttgcgt
 1901 gcaggtttt gcatgtctgg gtgagttgg tttgtgtgccc tgccctctg
 1951 ccagggcgtg gcgaggtgag gggcacggct tctccccaaa ggccttgcgt
 2001 agccctggcc tcccttcaag gagtcttgg gatgcctgct ctggctttt
 2051 tttaaaaaag tatctatttt atttatttt atttggtaa aaatagagac
 2101 agggctcac tatgttgcctc gggctggct caaagtccctg gttcaagca
 2151 ttcctctgc ctcagcctcc gaaagttctg ggattacagg catgagccac
 2201 cactccccgc ctgctctagt ctttgcatac ctagaggaca gtatggatac
 2251 aaaaaactt actccccacc aaccggcggaa gacagagtct tgctctgc
 2301 cccagactgg agtgcataatgg cggccatctg gctcaactgca acctccgc
 2351 cccaggttca agcgattctc ctgcctcagc ctcccgagta gctgggatta
 2401 cgggcacgc ccaccacgcc cagcatatgg tatttttagt agagacgggg
 2451 tttcaccatg ttggccaagc tggctcgaa ctctgaccc cgtgatccac
 2501 ccacctcgcc ctccccaaagt gctgggatc caggcgtgag ccaccacgcc
 2551 cggctggat acagaaagct ttatttcat cactgttcc tgcctggtgc

FIG.2A

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2601 caggcccattg ctggggttcc tcccaagtgg aattactgac ttaacattta
 2651 gcttgggatc ctgagacttc catcacacag ttttctcatt gattcgac
 2701 caataatatac tgttttaaaa acatctcagg ccgagcgtg tggctcacac
 2751 ctgtaatccc agcactttgg gaggctgagg tgggcagatc acctgaggtc
 2801 gggagttga gaccagcctg accaacatgg agaaaccctg tctttctaa
 2851 aaaaatacaa aattagccag gcgtggtggc gcatgcctgt aatcccagca
 2901 ctttgggagg ctgaggcagg agaatcgctt gaacccagga gacggaggtt
 2951 ccggtgagcc gagatcgccg cattgcactc cagcctggc aacaagagca
 3001 aaactccgtc tcaaacaacaa aaacaaaaaa catctctcg ctccttggg
 3051 ccgggtgcca gctctgctat tggaggcact gagcgcaccc ttggcagggca
 3101 tgtcactcct ctgtccccca gtttactcat ctgttaagtgg gggagactgg
 3151 ggcagacagt gagctggctg agggcaggac tgggtctcctt caagccatg
 3201 gcccagggtc gccaggtatg agtttgtatt cggttaatgc tgctggcccc
 3251 taagtgttag cgtccccctgc aaactgcagg gatgggtggg acagccctgc
 3301 acggctaccc ctttcttggg tgaccttatt tggttacggt cctatctgaa
 3351 gtaggaaagg gacacttttag gctgtctt agctccctca agggccac
 3401 gcctggacta gagttgcccag aaatacttgg tccattcagg ccaaaggag
 3451 tgtgagggtt ctgggatggt gcaatcagtc tttgtccatg atgaaccac
 3501 agggtagacc aggggttggg ccagcccaatg gccctgtgtt gttgagccca
 3551 ggccccaggc atccatccc gggcggtggc ctcaggtgg ggtggggcag
 3601 ccagttgcca gggatgtgtt ccagcggtca cctctcacca gccccggctg
 3651 cccatcagct gttctcaagt ccaggcaatg aaggcttcct gccagggaaat
 3701 tcccagagtt tctgtgccat gaagtcagcc tggccatc ttggacaca
 3751 aggccgggtg ccctggggag agtactctgg gccccttggcc aggtttgtct
 3801 gagagtataa ggcagcctga tactagtgg gccagccagg gagggtatgag
 3851 gcccagccgc tgctggccat aagtatataa gggccatgtg ctgagtgcc
 3901 actatgtgcc aggtttgaa atcagactt gatttattga aaccctctct
 3951 ttaatcctc aagggtcccc tatgaggcac gtaccattha ttgttattgc
 4001 cacttgcacag atgagaaaaac agaggctcag agaggccaaag tggcttgaaa
 4051 ttcaatgtt ggtctgggat ttgaatccac agccatgtt ttaagggcat
 4101 gctatgtgc cacctatcct gtttatttcc ggcactcatt gatttttcaa
 4151 tgtttgcattt attaaatcca tcagttagca tcttctctgt gtcatgcatt
 4201 gttctcacct ctgaagatgt agctgtgagc aaaacttcta cagggatgaa
 4251 gttcacagca gagggtatcag ctagagcaaa ggctcagagg tgggaccgtg
 4301 cgtcctgtgt tccaggaata cagtatggct gcagcagaga gcagtggaga
 4351 gagggcctgg cagttaggtc tagaggcggc cgggctggct catgctggat
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 4451 cccagagagc acagcaggaa agcctggga gtctgatgga catttaaaag
 4501 gatccttaat ggagagatgt aaggcagagc cttccagaag ggtaagagaa
 4551 gggaggatgg agacactgccc tcccccaagg gaggccactc agaagaggt
 4601 gagttggcc agggcagaga gcaagagagg ctgtggacac aggcacactg
 4651 gtcccaatgtt agccatttgc cacatttagat ttagcttcattt gttgtcttta
 4701 gagagggagc cagcctggcc tcgcctctatg atcttggaca catccttca
 4751 cttctgggtc tcagtttccc catttagtgg atgaggatga gaatgcttt
 4801 gtcctggca cactatgagg gtgggtctgg gcacctgggt gcctggttac
 4851 catgggcaac aaagctctat tcatgggtt ggtgaatgca ttgcccacag
 4901 caactcaggg cggatgagga gtttcccagg agcccctgtt gccctttcgg
 4951 ctgaaggccct aacaactgtt ggaaaatcca agttccagca gacccctgaa
 5001 gccccttgcc tttaggaccct ctttcttagt gttctctgtt gcctggctg
 5051 agctggagga gggagttggc agtgctgcag cagaggctgc ttcatagtaa
 5101 ttgcagccaa cagttattga cttaggcactt ttctgagggg ttttagatgt
 5151 gtaactgattt gaattcgccct aacaacttta tgaggttaatg cctattgtt
 5201 gcccattttt tagatgagga gactgagttt gaaactgggg ggtgtaatgg
 5251 aaccccttcata ggacccttgc agggtagggc ctttgactc gggccacgag

FIG.2B

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5301 ggtggggttt gtgtctgggt gggagctggg gagggacagg actaggatta
 5351 ggcagatctg aggccacagg agttgggtgg ggggtggctc cagagccact
 5401 ccactccctc ctaccacatt gactgccttg aaagtcccct aatggccact
 5451 cccatgaagt gtgactgctc tgggctcccc gcagggcggtt tctgcaaggc
 5501 caccgcccac ccagggccct tccccagagg ggctgcagtg cttgctcct
 5551 tccttgtggg aagagttggg attgtctggc gtcagcagga tactgcccct
 5601 gggcatccct cccggctct tcctgcgggt ttctgatgaa acagccaggg
 5651 tccagtagtg gagccagagg tcagtggtgg agagaggacc aggagccaga
 5701 ggttatagtc gctttggggc tactgtgggg tcagggacac ttgtgaggcc
 5751 aagcgtcctg gctgcaggag ccctcacata tatgccacc cttcacagg
 5801 acattgaggg gtgctggggc acagggtag ctttttgggg gtgtctgcct
 5851 tcgacttggg ctccgctaca caggccaaat ttggatgtcc catgtttaga
 5901 gctgtgttcc tttgggacct cttggggcct cagtttccctc atctgtaaaa
 5951 tgggatactg atagtgcttc cccactggcc tcctctgacg ggcgccaggg
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 6051 ccacctggga gaggggagag gcaggaatgt cttgggggtg tcctttgagg
 6101 catagccctg tcaccccaac atcctacaaa ggcatgagaa ggcagcgagg
 6151 acagaccccg accaccttag ccctcagcag ccctgcacaca ctcctgtcctt
 6201 caccccttc ctgactgatc tggcacattc ttgattctcc tagggagtga
 6251 cccaaaatcc ctccctgccc tgctgtgtct ctgggggtgga aggaggctgc
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 6351 gcagagaagc agcttctcca ctctcttccc tgacacctgt agggccctcc
 6401 tgcaggact tacctctaag tggactctca ggaggaggct catcagggtc
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 6651 ttcatcctg ggcaggctgg ctctgggaca ccccccggac cagctgtgt
 6701 gtgggattca cgggtggggct tggcaggggc gtgggatctt ggggccaact
 6751 gagccactct aggcttccag ggaccaaggc caggctgagc tgtctctgta
 6801 tcctgagaga gcatgaacat cacagaagat gggcccccgggt tcgaatccca
 6851 gctctgccac tactaactgg gacctggca ggggtccctt cccgctgagc
 6901 cttcatttcc tcaccagcaa aatggttcgt gcccctgctt tggggctgt
 6951 ggaggggtgg ctctgtcta cttgttcata cctgctgttg agcagctgct
 7001 ctgtccggc ctctgaggat gccactgtga acagagcctg tcgctaccc
 7051 caggagctt gtttagggg tgccgtttt attccagcac tttcaccagg
 7101 ctctgctccg gtacccgatg agagacgtcg agtcccgctt tccactcgct
 7151 tgggtgcgtg tgggggttgg ggggacaggg ctttgtgcac gtagccctgg
 7201 gtggatgttc ctgggtgcac ttaggggtgt tgaggggtggg acctccaca
 7251 gttcccttag gctccactga tgaggtccaa gaaccgcctt cctgcccccc
 7301 agccccaggct cccagcagct gggcccttgg cttcttgaga tagtactgg
 7351 cctcacggca aggacccccc cacaccaccc aggagaactg ctgcttcccc
 7401 tctgttccag gagtggcgac aagcacagtt tttcgctttt gttttgttt
 7451 tcttcacttt aagttccggg aaacgtgcag aatgtgcagg ttgttacat
 7501 aggtatacat gtgccatggt gtttgcgtc acccgtaac ccctcatcta
 7551 gttttaagc tccatataca tttagcattt gtcctaattgc tctccctcc
 7601 ctggcccttc acccgccccag taagccccgg tgggtgtatgt tcccttccct
 7651 gtgtccatgt gttctcattt ttcaactctc acttatgagt gagaagagac
 7701 ctggactctg atctaaccctc ggtcaaatgg aactgtgtga cttgaagaa
 7751 gtagcttaac ctctctgagc tttagctct gcctggcacc cccatcctta
 7801 aggagaggcc cacagaggac caggtcacat gacctcagcc agttccagag
 7851 aaggctgttt gcttccaggt ttggcctga gtccaggccc ctgcccact
 7901 cgcaactccct gatagcatga gaagcacagc cccaggggtgc ccacccagct
 7951 ctgagagccc agcctgcttc ccagggaaact gtcacagcccc cacctgtccc

FIG.2C

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8001 ttccccagct ggagccctgt caatggcttt ggggttctct gacacagccc
 8051 tgagggggct cacacttccc cttatcattg caagggttag atctggcttg
 8101 aaggccctgg ggcaggctt gttctgtcct cccctgtcag tgcctcgaca
 8151 gggctggcct gggtaatca ggaccaacgg gaaaggaggc gaggagacca
 8201 atctggaccc aagatcctca gctcaataag gtggcccccag aactgacatg
 8251 gggtgataga gggaaagggt gggaggggagg agattctggg gccgcagcca
 8301 cagcttgcac gttgcggcgg gtgtgtctgt gcgtgccagc tgcatcttg
 8351 cgtaccatgt gtgcaaggct gtgtttggct gagtgttcat gtgggcgtg
 8401 attgtgggca tggttcttag tggctgagtg atgcctgctg gtgtggctg
 8451 gtgggtgtgt ctgcatgtgc gtgtgtgtct ggggagtttca aaggagaaaa
 8501 gagggactca ccatcacgct ggctcagcct taaaaaggtt ggacatcctg
 8551 acacgtgctg caacatggat ggacctaag gacattgtgc tgagtggaaac
 8601 aagccagagg caaaggaaca aacatgttat ttctccaga tgaggtttcc
 8651 ggaggaggca gatctgtatg gacagaaggt agcatggtgg ttgcccggc
 8701 agggggagga gagaatggag aattagtgtt taatggggac agagtttcag
 8751 ttggggagg tggaaagggtt ctggagctgg atgatggtga tgggttgaca
 8801 acactgtgca tgcacttaat accactgagc tggacaccta aaaatgctta
 8851 caatggtaaa ttcatgtat atttactac aattttaaa aaattggctg
 8901 ggcgtggtgg cttatgcctg taatcccaac actttggag gccaaggccg
 8951 gaggattgtc tgagctcagg agttcaacac cagcctggc aatatggtga
 9001 aaccccgact ctacgaaata tacaaaaatt agcctggtgt ggtggcttgc
 9051 acctctaattt ccacctactc agtaggctaa ggcacaagaa tctcttgaac
 9101 ctgggagggtt gaggttgcag taagccgaga tcatgccact gcaaccagt
 9151 ctgggcgaca gagcaagact ctgtctcaaa aaataaaaaga taaataaaaa
 9201 aatttagaggc caggtgtggc tcacacctgt actctcaaca ctttgggagg
 9251 ctgaggtggg aggatcgctt gaagtcagggc atttaagaca tgccttaggca
 9301 acatagttag accttgactc tacaaaaaaa ttcaaaagtt aatgagacat
 9351 ggtggcatgt gcctgtagtc ctgactgctg gggagggctga ggtgggagga
 9401 tcacttacga ccaggatttc aaggctgcag tgagctgtga ttgcataact
 9451 gcactccagc ctggtgacag agtgaggccc tgtctcaaaa aaattttca
 9501 gtgttttctt gggctggcgg tgggtggctca ttcctgtat tccagcaactt
 9551 tgggaggctg aggtgggtgg attgctttag cccaggagtt taagaccagc
 9601 tgggcaacat ggcaaaccctc atctctacaa aaaataaaaaa taaaaaatta
 9651 gctgggcattt gtggtgacaca cctgtactaa cagctacgag agaggctaag
 9701 gtgggaggat cacctgagcc cgggagggtt aggctgcagt gagccatgat
 9751 tgcaccactg cactctagcc tgggcgatac agcaagaccc tatctcaaaa
 9801 aaaaaaaaaa aaaaaaaaaa aaaaacaccc agtgggtca gtagaaccac
 9851 aagagtcttc ttccctccca gctccccctgt acaccagccc cagctctgca
 9901 ggtagctggg ggcccagaca gcttctggg gaccccccag cttcaagact catgtccaga
 9951 cccctttttt taccagttt gctccccctc ttcaagact cttcaagact
 10001 ggggtgaga tctgcactta tacagcccccc tcctctgtaa tgagtggccc
 10051 aagtcaagccc aggttattcc agaaggggca cccttaccagc cccccagttc
 10101 ccaagctgcc ctgggcattt aaaagcaggc aaggggaccc ctagtagatc
 10151 atgttaggtt tacctcttag tgggtgctgg agggggctga agtgctttct
 10201 tccccccagg tggtaggaga atgtcttgcg agtgacttca gggccgcgtg
 10251 tcacttccgt tttaagactc accagctgtt aggctcatta gcaagaggac
 10301 aataggagggc ccctgtccctc agtcaagctt cttcaaaaggt gttcccttta
 10351 gcaactggga ggcctccctt ctccagaccc atggggacaa caccacccag
 10401 ctactggttc tataagctgc tggatggctc tggctagccc attcagagaa
 10451 agcctctgaa agtacaagga aaaaaatcag tccaagagct gtgaacaatt
 10501 agtgagccga ttacaataacc aagaccacag gcagacctgg aaggctaagt
 10551 gagcccaggt gtgaagttca agcttacttt acttctggc cacttcctgg
 10601 ctggtctttt tccctggccc ttatctttctt cctggtctgt cttctttct
 10651 cccccctttt ctttactctt tcttccttctt cctgcattc actccacccc

FIG.2D

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10701 cactccagct attacacaga atcgcgagaa tgttggatta ttcattttat
 10751 ttatgatgtt ttctttttttaaaaaataga gacaaggctc cactatgtgg
 10801 cccaggctgg tcttgaactc ctggcctcaa gcaatcctcg tgccttggcc
 10851 tcttacagtg ctgggattac agatgtgagc caccatgcct ggcccatttt
 10901 atttactta aaaaaaaaaat taggctgggc gcggtggctc acacctataa
 10951 ttccagcaact ttgggaggcc aagggtggca gatcaactga ggtcaggagt
 11001 taaagaccag ctggccacc tggggtcagg agtttgcagc cagctactcc
 11051 ggaggctgag accggagaat tgcttgcacc caggaggttag aggttgcatt
 11101 gaactgagat catgccatttgcatgcccagcc tgggcaacag agcaagactg
 11151 tctcaaaaaaa aaaaaaaaaat atgtttgttgcctcttcctgatccttgc
 11201 agtcaaatacgtttaactgt tcaagtgtct tccttgcataa ccccaagga
 11251 ctcaatgtgt gtcgccttgcactgatccccccgcggccctgt acccagtgg
 11301 cctcagttcc aggtttcccacctacccttgcacccactgc ttatgtttat
 11351 aaaaacgggg taaatcaaattgttgcgttgcacc cagatcttat tctacatgca
 11401 gtggaaactt gtatgactta agcttttggaaaaggcagaa cctttttcg
 11451 tggttcaaga aatcaaagtc ttcccgggag gtcttctgt aaatccagag
 11501 ctgcagatgt ttgaccgtgt tcagagaggg gcccctgtgc tgggtgaagt
 11551 ggtatggggca cagcaggccaa tgggtgaaaaa gcaggacaac ctggggccct
 11601 gggaggacca gggaggggccc atgttttgcgttgcataa cccctgtgc
 11651 ttcctgtccg cctgtcgcttgccttgc tccatccgtatcgttgc
 11701 ctgtctctgc tgggttgcgc tggctactc agctgtgtct gtctgtccgc
 11751 ctgactgtcttgccttgc agGATGCCTT CCGTGCCTTC CATCAAGATC
 11801 TCAATTTGT GCGCAAGTTC CTACAGCCCC TGTTGATTGG AGAGCTGGCT
 11851 CCGGAAGAAC CCAGCCAGGA TGGACCCCTG AATgtgagcc agagccctag
 11901 gagaggctca gcccctgagg gaggggatg gctggaggc tgggagacat
 11951 tgccacatgg ccaggagcag ctccctcgcc attcgcccaa gggatgcag
 12001 agccagggtct gaggccttgc tccctccca gggggcaggc agttgaaagt
 12051 gaagctgttag ggtatggccctg agaagtcag ggctccagat ctggtttagc
 12101 caggcactcg ttggatccc gaggcaagct ccctccctgt tgcggccag
 12151 tgcgtccatc aaaaggagga tttgtatgaa ctgatttctc tcctggctgt
 12201 agcgtcttac ccacccata ccttttggga gggagaggag gcttcaccac
 12251 cagccagtgc tccagctcac accccgggct ggttactctt gtcacttcat
 12301 tcctcttgc ccacacccct tgggcctggc gatgggagga gcggctgggg
 12351 ctccaggaga atgggggtgg gggaggatt ctcccttggc tgatggcc
 12401 ctctgctatg gcagGCAGCAG CTGGTCGAGG ACTTCCGAGC CCTGCACCA
 12451 GCAGCCGAGG ACATGAAGCT GTTTGATGCC AGTCCCACCT TCTTGCTTT
 12501 CCTACTGGGC CACATCCCTG CCATGGAGGT GCTGGCCTGG CTCCTTATCT
 12551 ACCTCCTGGG TCCTGGCTGG GTGCCAGTG CCCTGGCCGC CTTCATCCTG
 12601 GCCATCTCTC AGgtgacccc agttctgtgt tgcaagccacc ttaactgc
 12651 aacagacgtg gccccccatg catctggca ttgtgaacat atttgcataaa
 12701 tgaatgaatg gacctatgaa agatgtatg gatgaataaa cagatgtatg
 12751 agtgaacagt ctgaaggccc atcaggcatg tctgtgggtc aagctgcatt
 12801 ccagatgagc caagaaggcc tctcttgcac agattccgat caagcacagg
 12851 gcaactgagc cagaggctgc tgccctgcag cttcatgaca cttacgagcc
 12901 cctccacccctc cttgggactc agttctcatc tgaaaaaga ggacactggc
 12951 ccacaagggt cttgaaatgg agcattagca cgggggtacc ctgcaagctg
 13001 aaaggattca ctggggccccc aggccctggc gggctccgtc ctccccaaaca
 13051 gcttctgacc ctgcctctct ccccaagGCTC AGTCCCTGGTG TCTGCAGCAT
 13101 GACCTGGGCC ATGCCTCCAT CTTCAAGAAG TCCTGGTGG ACCACGTGGC
 13151 CCAGAAGTTC GTGATGGGGC AGCTAAAGgt gagggtgggg tgggtggca
 13201 gccaggtgct gggtggcgct gggtctggcc aagtgtgtgg gcacagtccg
 13251 gggcacagcc tgccctgaga gccccctcttgccttgc cttccacagG GCTTCTCCGC

FIG.2E

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13301	CCACTGGTGG	AACTTCCGCC	ACTTCCAGCA	CCACGCCAAG	CCAAACATCT
13351	TCCACAAAGA	CCCAGACGTG	ACGGTGGCGC	CCGTCTTCCT	CCTGGGGAG
13401	TCATCCGTG	AGgtgggtgg	ggagggacct	ggacaaccc	tggctggcc
13451	tgcagctgag	ggggagctaa	tgcactgggt	ccccactctg	cccctgaccc
13501	agccccgtat	ctggcctcca	ctctggctgg	gcacaagctct	gccccctgtgt
13551	ctttccctcc	cacccccc	cctgctgggg	acgaccagcc	cgcttgctag
13601	aatcttagat	tgcctttgac	ccttggcccc	agccagcccc	gtgacccctgc
13651	ccgggagaaag	gaggtggcct	ggagagctgc	tgtctccagc	cgccgcctgt
13701	ctccacagTA	TGGCAAGAAG	AAACGCAGAT	ACCTACCCTA	CAACCAGCAG
13751	CACCTGTACT	TCTTCCTGAg	tgagtgtcca	tctgtccttc	tgggtgggg
13801	gagtgcctgg	gcctgcactg	tcctccctgc	tgtcctggac	cactcccac
13851	cacttcctgg	ggcggggcac	gtctgtcagg	tctccctgggt	catggcatcc
13901	tcccagccctc	tgcagtctgt	acacactctc	ccagcagcat	gccttgc
13951	cagctgtctc	ccgtgcctgg	gacaccttgc	agccacggc	catcacagcc
14001	ctgctggag	cttcccaag	ccccacgtag	aatttcttct	tgcctctact
14051	agagtggtcc	ggagccctag	agtctttggg	cagttgttgg	ggccggacaga
14101	gtgaggactc	aagtctggcc	ctgacttgcg	gtgaagggtg	gtgggaggtg
14151	gtggggtaag	ggcagcctgg	ggaggcttgg	acacagaatt	gggggtgata
14201	tggggtcatt	cagctggatg	tgaccagcac	caacgtccca	ggggcattcc
14251	tggagtaaca	gagccctca	ctctggcgcc	cactcaccc	ggcagccag
14301	ccccactctc	gaacactctc	atgcccccttc	ttgcagTCGG	CCCGCCGCTG
14351	CTCACCCCTGG	TGAACTTGA	AGTGGAAAAT	CTGGCGTACA	TGCTGGTGTG
14401	CATGCAGTGG	GCGgtgagtg	gggttgccca	ggaccccccggg	catacggctg
14451	ccgtggcagg	agggtgggcc	tcgggggaca	gtacactgccc	atgaaggca
14501	acagggtgca	catgtgcgtg	caacagtgt	gctcacatgt	atgcgtgca
14551	cagtgtggct	cacatgtgt	cgcgcagcc	gagagcgt	gtgcccgtga
14601	ctgtacgtgt	ggtggggggg	ggttgaggaa	caggggggggt	gtgggtctct
14651	ctcggtgagg	gtgtcttccc	aggaggagtt	gctggggcca	ctctgcccagg
14701	catctgtgtc	cctggcaggg	tcttcccaa	cacaccctgc	atgacaccc
14751	cgtcactaaa	atcagctctg	ttagctggca	ggcaaggac	cctgttcctt
14801	tactcagctg	agaaaaccag	agagggttgt	ggctgtcct	gggtctgag
14851	gcaaattcagg	cagaagggtt	ggatgcctga	ggtctccctc	ccacccacca
14901	ggcctccaga	cctccggca	cctggagacc	tctcggtatc	gcctctgccc
14951	tcctctgcag	GATTTGCTCT	GGGCCGCAG	CTTCTATGCC	CGCTTCTTCT
15001	TATCCTACCT	CCCCCTCTAC	GGCGTCCCTG	GGGTGCTGCT	CTTCTTTGTT
15051	<u>GCTGTCAGgt</u>	atggcaggga	gtggcgaggt	cacacacagg	cgacagggtga
15101	cccccaactc	agccccccac	cagacttcc	ctttccctgt	ctgcagaatg
15151	ggcccaagtgg	tactgcctcc	ctggcttgct	ggtggaatca	cataaacaca
15201	agcgtggcag	gagcccaagg	tcgggtgggtt	tagggagcgt	ggcctggctt
15251	gtaagtggcc	cggtgggtgt	cggagctgt	ctggacttag	cctcacatgt
15301	gacactgctc	cattcagatt	ctttaaacac	tggcaagggg	gcgtatggcca
15351	caatcctatt	gtacagataa	ggaagtcaag	gccacttggg	gacagctgt
15401	ctccagccctc	cactcagggt	gcctaagtgg	tgagctggac	ctagggcagt
15451	ggccgagcc	ccccacagGG	TCCTGGAAAG	CCACTGGTTC	GTGTGGATCA
15501	CACAGATGAA	CCACATCCCC	AAGGAGATCG	GCCACGAGAA	GCACCGGGAC
15551	TGGGTCAGCT	CTCAGgtggg	cagcagggtt	ggggcccatc	ctgggtgggg
15601	tgggggtcc	cagctaggag	ccagatggca	aagcagggtat	gaggccctg
15651	cggggctgcc	agggtggggaa	tggtgcctg	gggtcaggga	tctgcaacgg
15701	cctccctaca	tgtccccgc	cggcttccgg	cagCTGGCAG	CCACCTGCAA
15751	CGTGGAGCCC	TCACCTTTCA	CCAACCTGGTT	CAGCGGGCAC	CTCAACTTCC
15801	AGATCGAGCA	CCAGttagtg	tgggtgtcgg	ggccagttgg	gaggtgggg
15851	gggggtcctg	ggagggatc	ctgggagggg	accctgtgggt	ggggcctctc

FIG.2F

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15901 tctggaatct cccacttcag gtgccagcat acgctccccca ccccccagCCT
 15951 CTTCCCCAGG ATGCCGAGAC ACAACTACAG CCGGGTGGCC CCGCTGGTCA
 16001 AGTCGCTGTG TGCCAAGCAC GGCCTCAGCT ACGAAAGTGA GCCCCTCCTC
 16051 ACCGCGCTGG TGGACATCGT CAGgtgaggc tgcagcccg cccctctgtt
 16101 ctgggtggctt ccccaaggcc tatgcctacc cttgtccagg tcagccctat
 16151 gctgagcccc cagggtccct gagccttct gtccacgtcc catgcccttc
 16201 ctccccttccc cagccttcac gcacacagtg agaatttctg gagcacctac
 16251 tgcagactca caaacagcag tgcctgcgtt gagcaggctc atgcaaacct
 16301 accccccaaag gctgagggaa aaaagctaac agatccagtt tctcagaagg
 16351 aaacacttaa cagggactca taaacagaag ccatgtctca gggccgggtg
 16401 cggtggctca cgcctgtaat tccagcatt ggggaggctg aggtggccgg
 16451 atcacttgag gtcaggagtt cgagaccagc ctggccaaca tggtaaaacc
 16501 cccgtctctac taaaaaaaaaaa aaaaaaaaaaaac aaaacaaaaac aaaaattagc
 16551 tgggtgtggc ggcaggtgcc cataatccca gctacttggg aggctgaggg
 16601 aggagaatca cttgaactcg caggggcaga gttgcagtg agctgagatt
 16651 gtgccttgc agtccagcct gggcaacaga gcaagactct ctcaaaaaaca
 16701 aacaaaaaaaaa ccatgtctca ggcagccaag agtgggaca tccccctcaca
 16751 cggccctctag aaagaaccct ctatatacga agcttttagg gtgaacccca
 16801 tgcaggtggc tcttatgaac ctgggtgacca ctggaggtt gataagcgtc
 16851 tacaagagga gtttatctat gccatgagct tggcattcaag ggtcaagcat
 16901 cggtcatcag acagtttgc ttgaagatgg catggccctt gtagcaatgc
 16951 aggctctaga gagcttcctg cccctcttgc gctgatgttc cttccagcaa
 17001 agggaaacagc aagcaattaa aataacaat aagtacatta cagaagatgg
 17051 gcaaaaagaac aatgaaaagc cccctcaggtt ggggacaggg gaggggagggg
 17101 gggcggccag gcagggggcgg cagtttctaa ataggtggta gggggcggcag
 17151 tattgacagg ctgacgtgtc agcaggagca gggaggaggg gagaggcttc
 17201 gccacagggc catctggcaa agagcgttca ggcagaggcc acttgaccct
 17251 gaatgccaag ctcatggcat agatagccga ggcaggcatg caggcactca
 17301 gagaagggac acgccccggc tgcatttgc aaagctgccc ctactgggaa
 17351 tgactggcgg gcaggagtcg aagtggaaaa ggagagcaga ggacactgca
 17401 gccatccagg cgaggggtga tggggtctcag cccttgcgtt caccttggag
 17451 gtggggaaaca gaggccagat tccaggtctt atacctctgc gccttgc
 17501 acgctgttcc cttacttgg ttgccttcc ttccctgtgt ggtgttcaga
 17551 tgcccacttc tccttcatga tctctccctag cctgatgttc tgagccccctg
 17601 ccatttggca cagcccttta gagcgcctgg cacagggctt cctagcagat
 17651 tggacatt tctggctcca ctgccccata tcaggcccaa gatgggtgg
 17701 gcaggttcca cgtcctctct gtccttgggt tgcagcgcggc agcaggaggc
 17751 agcaatggag aactgggtgc aggagggaca ggcccaccca ggctcatgcc
 17801 tggacttggc cttggctgcc ctccagctcc cctacccgc acccgtcacc
 17851 cccgtctaga ttccatttcca gagaatgagc attcagctgt tctcccaacc
 17901 caccctccag cccgcattcgc tgcctgcggc cagggaaaggg aaccacagg
 17951 gaatggggat ctccgctcac acttaccatg ggggatacag ggggtttagg
 18001 atcttgcaac tgagctccta acacccaccc ccactgcccac ccccacctcc
 18051 cagGTCCCTG AAGAAGTCTG GTGACATCTG GCTGGACGCC TACCTCCATC
 18101 AGTGAAGGCA ACACCCAGGC GGGCAGAGAA GGGCTCAGGG CACCAGCAAC
 18151 CAAGCCAGCC CCCGGCGGGGA TCGATACCCC CACCCCTCCA CTGGCCAGCC
 18201 TGGGGGTGCC CTGCCTGCC CCCTGGTACT GTTGTCTTCC CCTCGGGCCCC
 18251 CTCACATGTG TATTCAAGCAG CCCTATGCC TTGGCTCTGG GCCTGATGGG
 18301 ACAGGGGTAG AGGGAAGGTG AGCATAGCAC ATTTTCCCTAG AGCGAGAATT
 18351 GGGGGAAAGC TGTTATTTT ATATTAAT ACATTCAAGAT GTATTATGGA
 18401 GT

FIG.2G

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1	CTTCGCTTCCCTCGGGGTCTTGCTCGGACCTCGGCCACCGCCTGGATCC	50
51	CCAGGACTCGTGCCTGCAGCATGGCGGCCGTCGGGAGCCGGACCGCGG	100
1	M G G V G E P G P R	10
101	GAGGGACCCGCGCAGCCGGGGCACCGCTGCCACCTCTGCTGGAGCA	150
11	E G P A Q P G A P L P T F C W E Q	27
151	GATCCGCGCGCACGACCAGCCGGCGACAAGTGGCTGGTCATCGAGCGCC	200
28	I R A H D Q P G D K W L V I E R R	44
201	GCGTCTACGACATCAGCCGCTGGGCACAGCGGCACCCAGGGGGCAGCCGC	250
45	V Y D I S R W A Q R H P G G S R	60
251	CTCATCGGCCACCACGGCGCTGAGGACGCCACGGATGCCTTCCGTGCCCTT	300
61	L I G H H G A E D A T D A F R A F	77
301	CCATCAAGATCTCAATTTGTGCGCAAGTTCTACAGCCCTGTTGATTG	350
78	H Q D L N F V R K F L Q P L L I G	94
351	GAGAGCTGGCTCCGGAAGAACCCAGCCAGGATGGACCCCTGAATGCGCAG	400
95	E L A P E E P S Q D G P L N A Q	110
401	CTGGTCGAGGACTTCCGAGCCCTGCACCAGGCAGCCGAGGACATGAAGCT	450
111	L V E D F R A L H Q A A E D M K L	127
451	GTTGATGCCAGTCCCACCTCTTGTCTTACTGGCCACATCCTGG	500
128	F D A S P T F F A F L L G H I L A	144
501	CCATGGAGGTGCTGGCCTGGCTCCTTATCTACCTCCTGGGTCTGGCTGG	550
145	M E V L A W L L I Y L L G P G W	160
551	GTGCCAGTGCCCTGGCGCCTTCATCCTGGCCATCTCTCAGGCTCAGTC	600
161	V P S A L A A F I L A I S Q A Q S	177
601	CTGGTGTCTGCAGCATGACCTGGCCATGCCTCCATCTTCAAGAAGTCCT	650
178	W C L Q H D L G H A S I F K K S W	194
651	GGTGGAACCACGTGGCCCAGAAGTTCGTGATGGGGCAGCTAAAGGGCTTC	700
195	W N H V A Q K F V M G Q L K G F	210

FIG.3A

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701	TCCGCCCACTGGTGGAACTTCCGCCACTTCCAGCACCGCCAAGCCAA	750
211	S A H W W N F R H F Q H H A K P N	227
751	CATCTTCCACAAAGACCCAGACGTGACGGTGGCGCCCGTCTCCCTCCTGG	800
228	I F H K D P D V T V A P V F L L G	244
801	GGGAGTCATCCGTCGAGTATGGCAAGAAGAACGCAGATACCTACCCCTAC	850
245	E S S V E Y G K K R R Y L P Y	260
851	AACCAGCAGCACCTGTACTTCTTCCCTGATCGGCCCGCCGCTGCTCACCCCT	900
261	N Q Q H L Y F F L I G P P L L T L	277
901	GGTGAACCTTGAAAGTGGAAAATCTGGCGTACATGCTGGTGTGCATGCAGT	950
278	V N F E V E N L A Y M L V C M Q W	294
951	GGCGGGATTTGCTCTGGGCCGCCAGCTCTATGCCGCTTCTTCTTATCC	1000
295	A D L L W A A S F Y A R F F L S	310
1001	TACCTCCCCTCTACGGCGTCCCTGGGGTGTGCTCTTCTTGTGCTGT	1050
311	Y L P F Y G V P G V L L F F V A V	327
1051	CAGGGTCCTGGAAAGCCACTGGTCGTGGATCACACAGATGAACCACA	1100
328	R V L E S H W F V W I T Q M N H I	344
1101	TCCCCAAGGAGATGGCCACGAGAAGCACCAGGACTGGTCAGCTCTCAG	1150
345	P K E I G H E K H R D W V S S Q	360
1151	CTGGCAGCCACCTGCAACGTGGAGCCCTCACTTTCACCAACTGGTCAG	1200
361	L A A T C N V E P S L F T N W F S	377
1201	CGGGCACCTCAACTTCCAGATCGAGCACCACTCTCCAGGATGCCGA	1250
378	G H L N F Q I E H H L F P R M P R	394
1251	GACACAACACAGCCGGTGGCCCGCTGGTCAAGTCGCTGTGTGCCAAG	1300
395	H N Y S R V A P L V K S L C A K	410
1301	CACGGCCTCAGCTACGAAGTGAAGCCCTTCCCTCACCGCGCTGGTGGACAT	1350
411	H G L S Y E V K P F L T A L V D I	427
1351	CGTCAGGTCCCTGAAGAAGTCTGGTGACATCTGGCTGGACGCCAACCTCC	1400
428	V R S L K K S G D I W L D A Y L H	444

FIG.3B

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1401	ATCAGTGAAGGCAACACCCAGGCAGGGCAGAGAAGGGCTCAGGGCACCAGC	1450
445	Q	445
1451	AACCAAGCCAGCCCCGGCGGGATCGATA	1500
1501	CCCCCACCCTCCACTGGCCA	1550
1551	GCCTGGGGGTGCACTGCCTGCCCTCCTGGTACTGTTGTCTTCCCCTCGGC	1600
1601	CCCCTCACATGTGTATTCA	1650
1651	GAGCAGGGTAGAGGGAAGGTGAGCATA	1700
	GCACATTTCCTAGAGCGAGA	
	ATTGGGGAAAGCTGTTATT	
	TTTATATTAAAATACATT	
	CAGATGTAAAAA	

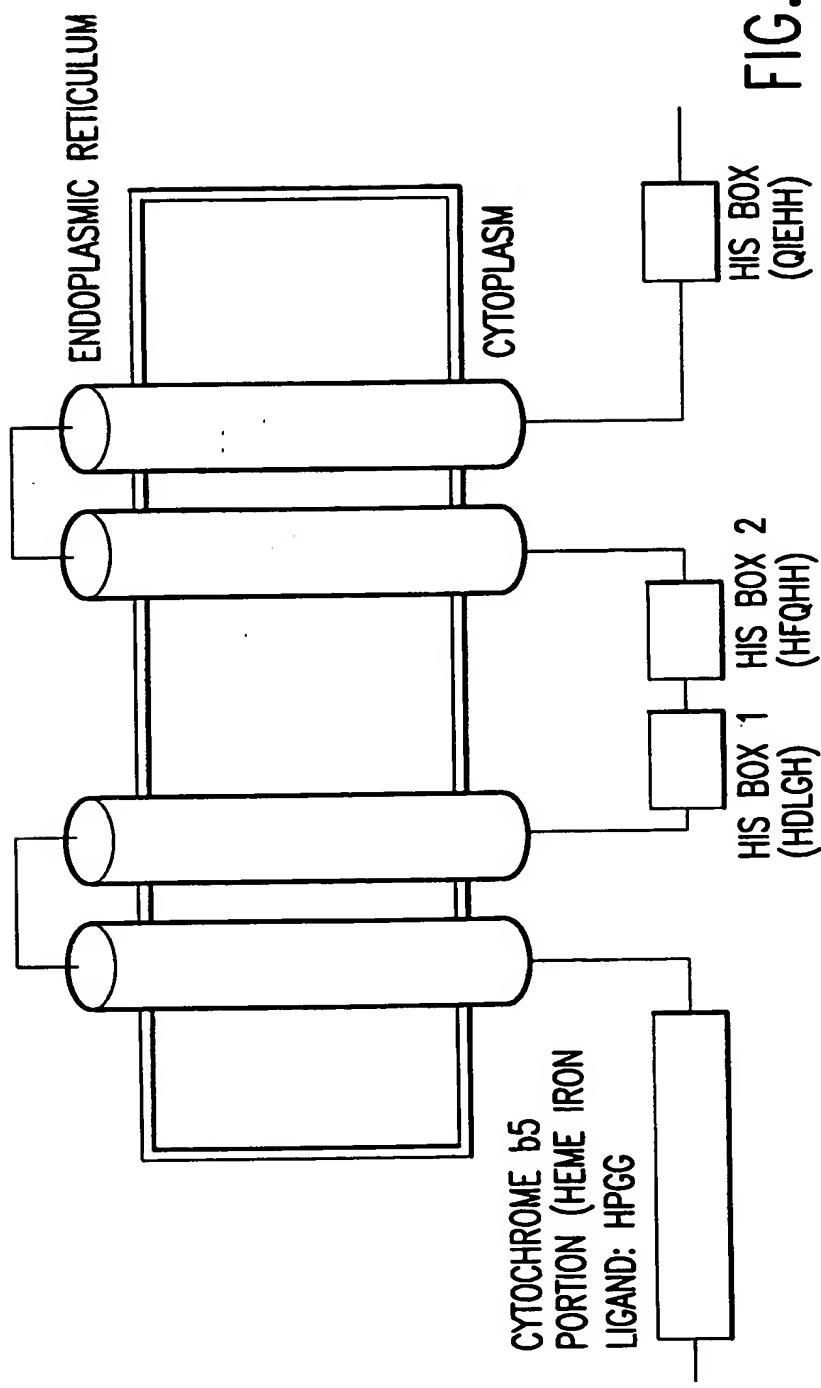
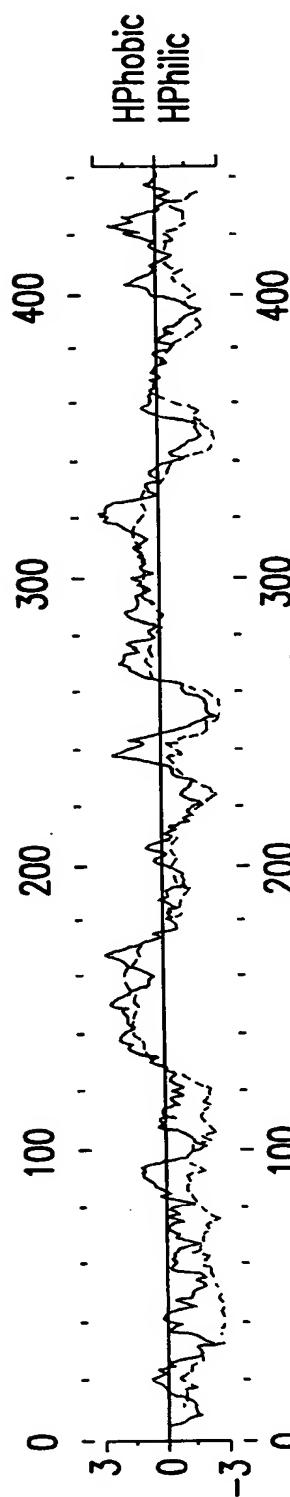
FIG.3C

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1	GTACAGCGGCAATGGGCGGTGTCGGGGAGCCCGGAGGGGGACTCGGGCCG	50
1	M G G V G E P G G G L G P	13
51	CGGGAGGGGCCCGCACCGCTGGGGCGCCCTACCCATCTCCGCTGGGA	100
14	R E G P A P L G A P L P I F R W E	30
101	GCAGATCCGCCAGCATGACCTACCAGGCACAAAGTGGCTGGTCATCGAGC	150
31	Q I R Q H D L P G D K W L V I E R	47
151	GCCGTGTCTACGACATCAGCCGCTGGGCACAGCGGCACCCAGGGGGTAGC	200
48	R V Y D I S R W A Q R H P G G S	63
201	CGCATCATCGGCCACCA CGG 220	
64	R I I G H H 69	

FIG.4

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PROFILESCAN of : CYB5rp_correct_protein check: 5714 from: 1 to: 445

GETSEQ from bmd, December 2, 1997 14:20.

Compare to profile library: GenRunData:profilescan.fil

Profile: profiledir:cytochrome_b5.prf

Gap weight: 4.50 Gap Length weight: 0.05

Ave match: 0.27 Ave mismatch : -0.21

(Peptide) PROFILEMAKE v4.40 of: 0191.Msf2{*} Length: 48

Sequences: 24 MaxScore: 27.58 December 2, 1992 00:07

This profile is derived from PROSITE release 10.0 and has been tested by a database search against SWISS-PROT release 26.0. A comparison of the SWISS-PROT annotation and the results of the database search follows. For further information about this motif, consult the . . .

Profile: profiledir:cytochrome_b5.prf alignment: 1

Quality: 20.77 Gaps: 0

Ratio: 0.43 Length: 48

Normalized quality: 2.91

S 31 HDQPGDKWLVIERRVYDISRWAQRHPGGSRLIGHHGAEDATDAFRAFH 78

 |: ..: ||||. .|||::| . ||||. | .||.||:||.|| ::|

P 1 HNDGEETWLVNGQVYDITKFLLEHPGGPDVIMEAAGTDATEEFAIH 48

*Cytochrome b5 family, heme-binding domain signature *

FIG.6

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pir:s68358 hypothetical protein - common sunflower
Length = 458

Score = 169 (79.4 bits), Expect = 2.8e-42, Sum P(4) = 2.8e-42
Identities = 31/85 (36%), Positives = 49/85 (57%) His box 3

Query: 348 IGHEKHRDWVSSQLAATCNVEPSLFTNWFSGHLNFQIEHHLFPRMPRHNSRVAPLVKSL 407
+G K +W Q T ++ S + +WF G L FQ+EHHLFPR+PR + ++P+ + L
Sbjct: 348 VGPPKGDNWFEKQTRGTIDIACSSWMDWFFGGLQFQLEHHLFPRLPRCHLRSISPICREL 407

Query: 408 CAKHGLSYEVKPFLTALVDIVRSLK 432
C K+ L Y F A V +++L+
Sbjct: 408 CKKYNLPYVSLSFYDANVTTLKTLR 432

Score = 133 (62.5 bits), Expect = 2.8e-42, Sum P(4) = 2.8e-42
Identities = 21/53 (39%), Positives = 35/53 (66%)

HPGG motif

Query: 26 EQIRAHDPGDKWLVIERRVYDISRWAQRHPGGSRЛИGHGAEDATDAFRAFH 78
++++ H+ P D W+ I +VY+++ WA+ HPGG + + +D TDAF AFH
Sbjct: 22 KELKKHNNPNDLWISILGKVYNTEWAKEHPGGDAPLINLAGQDVTDAFIAFH 74

Score = 118 (55.5 bits), Expect = 2.8e-42, Sum P(4) = 2.8e-42
Identities = 25/76 (32%), Positives = 34/76 (44%)

His box 1

His box 2

Query: 165 LAAFILAISQAQSWCLQHDLGHASIFKKSWNHVAQKFVMGQLKGFSAHWNFRHFQHEA 224
L+ IL ++ Q L HD GH + WN A F+ + G S WW + H HH
Sbjct: 152 LSGAILGLAWMQIAYLGHDAQHYQMMATRGWNKFAGIFIGNCITGISIAWWKWTHNAHHI 211

Query: 225 KPNIFHKDPDVTVAPV 240
N DPD+ P+
Sbjct: 212 ACNSLDYDPDLQHLP 227

Score = 34 (16.0 bits), Expect = 2.8e-42, Sum P(4) = 2.8e-42
Identities = 7/14 (50%), Positives = 9/14 (64%)

FIG. 7A

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gp:bou79010 1 PID:g2062403 *Borago officinalis* delta 6 desaturase mRNA,
 complete cds. (gb:U79010) (NID:2062402)
 Length = 448

Score = 179 (84.1 bits), Expect = 2.3e-42, Sum P(3) = 2.3e-42
 Identities = 34/87 (39%), Positives = 48/87 (55%)

His box 3

Query: 348 IGHEKHRDWVSSQLAATCNVEPSLFTNWFSGHLNFQIEHHLFPRMPRHNSRVAPLVKSL 407
 +G K +W Q T + + +WF G L F QIEHHLF P+MPR N +H P V L
 Sbjct: 338 VGKPKGNWFEKQTDGTLDISCPPMDWFHGGLQFQIEHHLF PPKMPCNLRKISPYVIEL 397

Query: 408 CAKHGLSYEVKPFLTALVDIVRSLKKS 434
 C KH L Y F A +R+L+ +
 Sbjct: 398 CKKHNLPYNYASF SKANEMTLRTLRLNT 424

Score = 144 (67.7 bits), Expect = 2.3e-42, Sum P(3) = 2.3e-42
 Identities = 23/53 (43%), Positives = 36/53 (67%)

HPGG MOTIF

Query: 26 EQIRAHQPGDKWLVIERRVYDISRWAQRH HPGGSRLIGHHGAEDATDAFRAFH 78
 + + + HD+PGD W+ I+ + YD+S W+ HPGGS + + + TDAF AFH
 Sbjct: 12 DELKNHDKPGDLWISIQGKAYDVSDWVKD HPGGSPLKSLAGQEVTDAFVAFH 64

Score = 105 (49.3 bits), Expect = 2.3e-42, Sum P(3) = 2.3e-42
 Identities = 22/68 (32%), Positives = 28/68 (41%)

His box 1

His box 2

Query: 176 QSWCLQHDLGHASIFKKSWNHVAQKFVMGQLKGFSAHWNFRHFQHHAKPNIFHKDPDV 235
 QS + HD GH + S N F L G S WW + H HH N DPD+
 Sbjct: 153 QSGWIGHDAGHYMVVSDSRLNKFMGIFAANCLSGISIGWWKWNHNAHHIACNSLEYDPDL 212

Query: 236 TVAPVFLL 243
 p ++
 Sbjct: 213 QVIPFLVV 220

FIG. 7B

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pir:s35157 Delta(6)-desaturase - Synechocystis sp.
Length = 359

Score = 126 (59.2 bits), Expect = 9.0e-09, Sum P(2) = 9.0e-09
Identities = 21/54 (38%), Positives = 33/54 (61%)

His box 3

Query: 372 FTNWFSGHLNFQIEHHLFPRM~~PRH~~NSRVAPLVKSLCAKHGLSYEVKPFLTALV 425
F NMF G LN Q+ HHLFP + +Y ++ ++K +C + G+ Y+V P A +
Sbjct: 292 FWNWFCGGLNHQVTHHLFPNICH~~I~~HYPQLEN~~I~~IKDVCQ~~E~~FGVEYKVYPTFKA~~AA~~ 345

Score = 36 (16.9 bits), Expect = 9.0e-09, Sum P(2) = 9.0e-09
Identities = 6/15 (40%), Positives = 8/15 (53%)

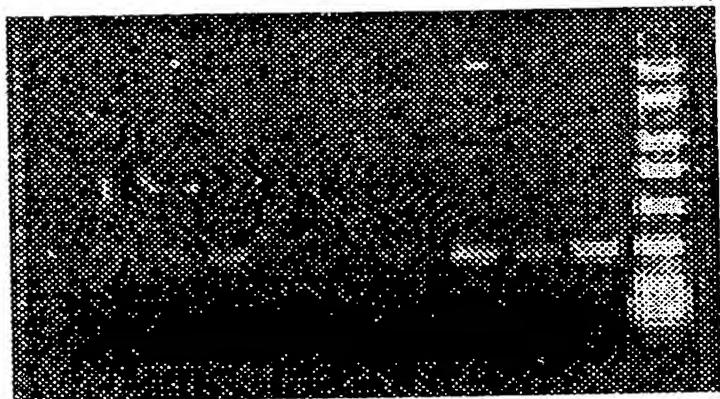
His box 2

Query: 209 GFSAHWWNFRHFQHH 223
G S+ W +RH H
Sbjct: 113 GLSSFLWRYR~~H~~NYLH 127

FIG.8

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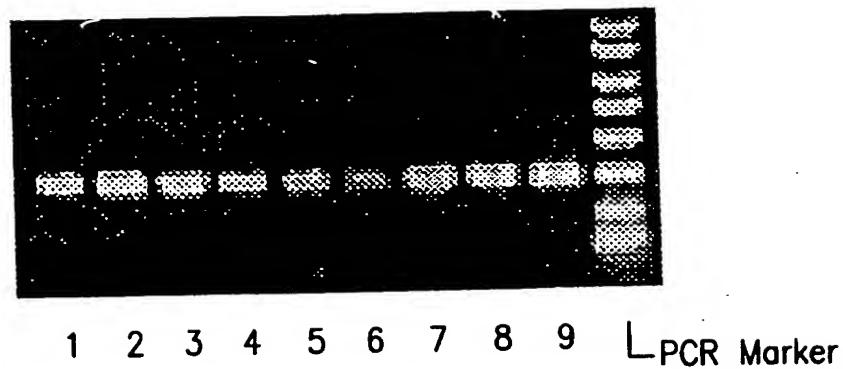
1 2 3 4 5 6 7 8 9



1. Heart	6. Skeletal Muscle
2. Brain	7. Kidney
3. Placenta	8. Pancreas
4. Lung	9. Retina
5. Liver	

FIG.9A

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1. Heart	6. Skeletal Muscle
2. Brain	7. Kidney
3. Placenta	8. Pancreas
4. Lung	9. Retina
5. Liver	

FIG.9B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23253

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 7/9395; C12P 7/62; C12N 9/02, 15/00; C07H 19/00
US CL :435/135, 189, 320.1, 452.3; 424/130.1; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/135, 189, 320.1, 452.3; 424/130.1; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Please See Extra Sheet.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Medline

Search terms: CYB5RP, delta-6 fatty acid desaturase, human or homo sapiens.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database GenBank, Accession AAC23396, submitted by LAMERDIN, JE, publicly available on 12 June 1998, see entire record.	1-15
X	Database GenBank, Accession AC004770, submitted by LAMERDIN, JE, publicly available on 12 June 1998, see entire record, especially identification of CDS at about line 50.	1-15
X,P	Database GenBank, Accession AAD31282 submitted by LI et al, publicly available on 19 May 1999, see entire record.	1-15
X	WO 98/39446 A2 (HUMAN GENOME SCIENCES, INC.) 11 September 1998, see entire document, especially SEQ ID No:63.	1-15

Further documents are listed in the continuation of Box C. See patent family annex.

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A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*&*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

24 FEBRUARY 2000

Date of mailing of the international search report

15 MAR 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

BRADLEY S. MAYFIELD

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No. PCT/US99/23253

B. FIELDS SEARCHED

Documentation other than minimum documentation that are included in the fields searched:

Because a CRF was not made available at the time of the search, Database GenBank Accession AF134404, which appears to encode the same desaturase as set forth in Figures 3A-C of the instant application, was searched against all available amino acid and nucleic acid databases.

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